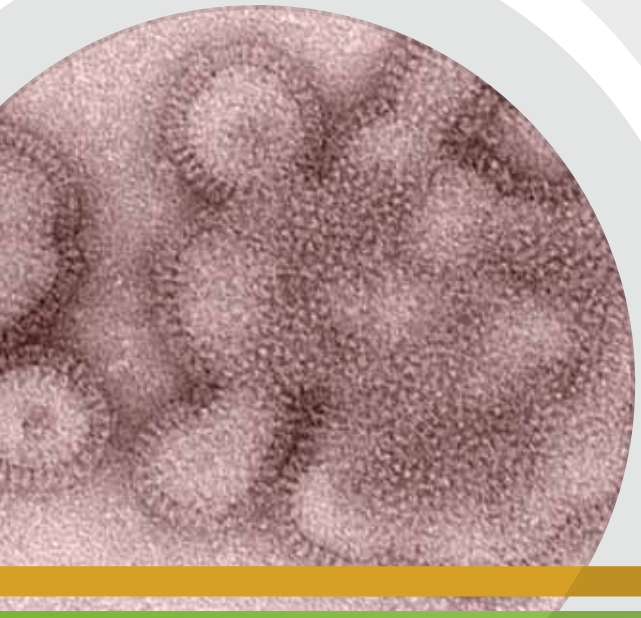


TECHNICAL REPORT

CNRL *in silico* exercise to determine the capabilities of network laboratories to detect triple reassortant swine origin influenza A(H3N2) viruses

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

CNRL *in silico* exercise to determine the capabilities of network laboratories to detect triple reassortant swine origin influenza A(H3N2) viruses

This report of the European Centre for Disease Prevention and Control (ECDC) was coordinated by Eeva Broberg.
Contributing authors
Joanna Ellis (Health Protection Agency), Maria Zambon (Health Protection Agency), Rod Daniels (National Institute for Medical Research), John McCauley (National Institute for Medical Research), Adam Meijer (National Institute for Public Health and the Environment), Ian Brown (Animal Health and Veterinary Laboratories Agency)

Acknowledgements The authors would like to acknowledge the assistance of Kate Howell (Health Protection Agency) and Nichola Goddard (Health Protection Agency) in collating the responses to the *in silico* assessment, and thank members of the CNRL for their participation in the exercise.

Suggested citation: European Centre for Disease Prevention and Control. CNRL *in silico* exercise to determine the capabilities of network laboratories to detect triple reassortant swine origin influenza A(H3N2) viruses. Stockholm: ECDC; 2012.

Stockholm, April 2012

ISBN 978-92-9193-344-0

doi 10.2900/36267

Contents

1. Executive summary

An exercise to assess the Community Network of Reference Laboratories for Human Influenza in Europe's (CNRL) capability to detect novel reassortant and circulating triple reassortant swine viruses (TRA) in humans was completed in July 2011. These are viruses that are appearing in the United States in pigs (swine) and are occasionally seen there in humans but have yet to be reported in Europe in either swine or humans.

A short questionnaire was distributed to 35 laboratories in 29 countries to assess the predicted detection of TRA A(H3N2) viruses using a theoretical, computer-based (*in silico)* approach. The objective was to identify gaps in the capability of laboratories and countries to detect and subtype such swine origin viruses, should they appear in Europe. Responses were received from all participating laboratories.

Type A influenza virus detection by real-time reverse transcription-polymerase chain reaction (RT-PCR) is performed by the majority of responding laboratories (94%) and countries (93%), using the matrix (M) gene as the target. Where predictions could be made (31 laboratories in 25 countries), the typing real-time RT-PCR assay employed was predicted to be capable of detecting TRA viruses. This predicts that the network has a good capability for detecting TRA viruses when using generic influenza A real-time RT-PCR assays.

Subtype determination of influenza A(H3) by real-time RT-PCR is undertaken by nearly all responding network laboratories (34) and countries (28). Prediction of the RT-PCR subtyping assays ability to detect TRA H3N2 viruses highlighted that this subtyping capability is significantly reduced compared to type A specific detection. Whilst this signals gaps in the CNRL network for identification of TRA viruses of swine origin, this overall position is as would be expected, with many different protocols in use for subtype determination, which requires the most highly variable genes to be targeted.

The *in silico* exercise provided a technical performance prediction of individual assays, rather than a reflection of how they are employed in laboratory algorithms, which may significantly affect the ability of a laboratory/country to detect unusual variants. If laboratories use a screening approach, testing initially with a generic influenza A real-time RT-PCR assay first, then the capability for detecting TRA swine origin H3N2 viruses is good. However, if laboratories use algorithms with a subtyping real-time RT-PCR assay detecting H3 and, or N2 gene segments first, then these viruses could be missed in approximately 30% of laboratories.

The following conclusions and recommendations were made from the results of the analysis of the *in silico* exercise;

- The exercise proved to be useful in highlighting development requirements and gaps in detection and identification capabilities for potential emerging influenza A TRA viruses of swine origin. Where countries have more than one CNRL laboratory, there is a requirement to relate the capability of laboratories to the overall in-country capabilities. Within Europe, there is a need to upgrade the capability for detection and identification of swine origin viruses.
- A review of algorithms for influenza virus detection and identification used in CNRL laboratories is required to determine the detection capabilities of individual countries and within the network overall.
- The *in silico* assessment should be followed up with a proficiency panel containing the same North American TRA viruses considered in the *in silico* exercise, to provide qualitative and quantitative experimental data to assess the validity of the *in silico* approach.
- The proficiency panel should be extended to contain more recent TRA H3N2 North American viruses, and Eurasian swine viruses.
- The discrepancy between high confidence in the ability of M gene RT-PCR assays to detect TRA H3N2 viruses but the lower ability of RT-PCR based subtyping, requires clear algorithms for rapid investigation of viruses which are not subtypable.
- Although a number of the H3 and N2 subtyping protocols are predicted to be positive with the TRA H3N2 virus, this would not identify the virus as being a TRA H3N2 virus. Therefore, testing algorithms should include the capability to distinguish seasonal human viruses from new zoonotic influenza viruses.
- Methodology for rapidly assessing laboratory protocols *in silico* should be developed through web-based sequence questionnaire tools, to enhance the rapid assessment of diagnostic capability within the CNRL network in the event of an emerging virus.

2. Introduction

Although aquatic birds are the major influenza A virus reservoir, swine play an important role in the ecology of human influenza. Swine support the replication of both mammalian and avian influenza viruses, hence they have been postulated to be a mixing vessel where avian and mammalian viruses can reassort [1–2].

Swine influenza is a highly contagious respiratory tract infection of pigs, which can have a significant economic impact, with low mortality rates but high morbidity. Swine influenza is enzootic in many areas with dense pig populations. Three influenza A subtypes (H1N1, H3N2 and H1N2) are currently circulating in swine populations worldwide [3]. The origin and composition of these viruses differ significantly between continents; thus Eurasian H1N1, H3N2 and H1N2 swine influenza viruses are genetically and antigenically different from their counterparts in North America.

Swine influenza was first recognised in pigs in the United States in 1918, coinciding with the human influenza A(H1N1) pandemic of 1918–19 [4]. The first influenza viruses isolated from pigs in the 1930s belonged to the H1N1 lineage of swine influenza viruses, and were the progenitors of the 'classical' H1N1 lineage of swine influenza viruses [5]. Although influenza A viruses of other subtypes (H3N2 and H1N2) have been isolated relatively frequently from pigs in Europe and Asia, influenza viruses of the classical H1N1 lineage were the dominant cause of influenza among North American pigs until 1998 [6]. Since then, new triple reassortant viruses containing influenza virus genes of human, avian and swine origin, including TRA H1N1, TRA H1N2 and TRA H3N2 subtypes have emerged and rapidly spread throughout the United States swine population, with TRA H3N2 becoming predominant [7-9]. Swine TRA H3N2 viruses contain HA, NA and PB1 genes of human influenza virus origin; M, NP and NS genes of classical swine influenza virus origin; and PA and PB2 genes of avian influenza virus origin. The H3 and N2 genes originated from human seasonal H3N2 influenza viruses that circulated globally among humans in the late 1990s. However, the H3 and N2 genes in currently circulating swine H3N2 viruses have evolved separately to their human counterparts and are now considerably different to the seasonal H3N2 influenza viruses currently circulating in the human population.

Human infections with swine influenza viruses are detected infrequently, and cases usually occur following direct or indirect contact with pigs [10]. In the United States, sporadic cases of human infection with swine TRA viruses have been confirmed, most recently with swine TRA H3N2 subtype viruses.

In November 2010, the United States reported through the International Health Regulations, two unlinked cases of swine TRA H3N2 infection, which were identified through normal virological surveillance [11]. Early identification and investigation of such human infections with novel influenza A viruses is critical to evaluate the extent of the outbreak and possible human to human transmission.

There were further reports of human infections with TRA H3N2 viruses in the United States in 2011, and as of 31 December 2011, the total number of confirmed cases of human infection with swine origin influenza A viruses in the country since 2005 is 35; 20 TRA H3N2 viruses, 13 TRA H1N1 viruses, and two TRA H1N2 virus [12].

As for any other unusual influenza viruses infecting humans, it is important to detect such infections as early as possible. This relies on European laboratories having the capability to do so. The exercise described in this paper is a step towards facilitating this goal.

3. *In Silico* exercise

Following the detection of two human infections with swine TRA H3N2 viruses in North America in late 2010, the European Centre for Disease Prevention and Control (ECDC) convened a teleconference with the CNRL coordination group to discuss the network's capability to detect reassortant and circulating swine influenza viruses in humans. As a result, the CNRL coordination group, with collaboration from the Animal Health and Veterinary Laboratories Agency, Weybridge, developed a theoretical, computer-based (*in silico)* exercise for CNRL laboratories to check whether their diagnostic primer and probe sequences for influenza A typing and subtyping would detect the relevant TRA H3N2 swine origin viruses.

A short questionnaire relating to the diagnostic RT-PCR assays used for influenza A typing and H3N2 subtyping was distributed in June 2011 to CNRL laboratories, together with alignments of representative gene sequences (M, NS, NP, HA and NA) of North American TRA H3N2 swine origin viruses associated with human or swine infections, as available in early 2011. Laboratories were asked to complete the questionnaire with information on which genes are targeted in typing and subtyping assays; the position and sequences of the primers and probes used; the reference/standard operating procedure (SOP) for the assay(s); predicted ability of the assay(s) to detect the viruses in the alignments; and any other comments or information they wished to include.

Laboratories based the predicted detection of TRA H3N2 viruses represented in the sequence alignments on the comparison of primer/probe sequences with their target regions.

4. Objectives

The objectives of the *in silico* exercise were;

- to determine the capability of laboratories and countries to detect the North American swine viruses by generic influenza A RT-PCR.
- to ascertain how many laboratories and countries have the capability to subtype by RT-PCR, the HA and NA of the North American swine viruses.
- to identify gaps in the capability of the laboratories and countries to detect, and subtype the North American swine viruses by RT-PCR.
- to ascertain if the responses inform a proposed second stage of the exercise, involving a proficiency panel which will evaluate diagnostic capability on a practical level, and allow assessment of the usefulness of the *in silico* approach.

5. Outcomes

A preliminary report on the responses was made and sent to ECDC in September 2011. The preliminary findings were presented during the CNRL virology task group's meeting in Prague, 13–14 October 2011, and the final analysis of the responses is presented in this report, together with recommendations.

6 Methods

6.1 Questionnaire

The questionnaire, (Appendix 1) comprised of an Excel worksheet for participants to complete their responses to the following questions regarding their real-time and conventional diagnostic RT-PCR assays for influenza A virus typing and H3N2 subtyping;

Part A Real-time RT-PCR:

i. Which genes are targeted in real-time RT-PCR assays in your laboratory for typing and subtyping of influenza A(H3N2) viruses – (M, NS, NP, HA, NA)

ii. Position of forward primer(s) & sequence

iii. Position of reverse primer(s) & sequence

iv. Position of probe(s) and sequence

v. Predicted detection of viruses in alignment

vi. Reference (publication/SOP) for assay(s)

vii. Other comments/information

Part B. Conventional RT-PCR:

The same questions as for Part A, with the exception of question iv, which is not applicable to conventional RT-PCR.

Alignments of representative gene sequences (M, NS, NP, HA and NA) of eight North American TRA H3N2 swine origin viruses associated with human (2009–2010) or swine (1999) infections, that were available in early 2011, were distributed as text files with the questionnaire (Appendix 3).

6.2 Participants

The questionnaire was distributed by ECDC to national contact points and influenza laboratory experts in 35 laboratories in 29 countries (Appendix 2).

7 Results

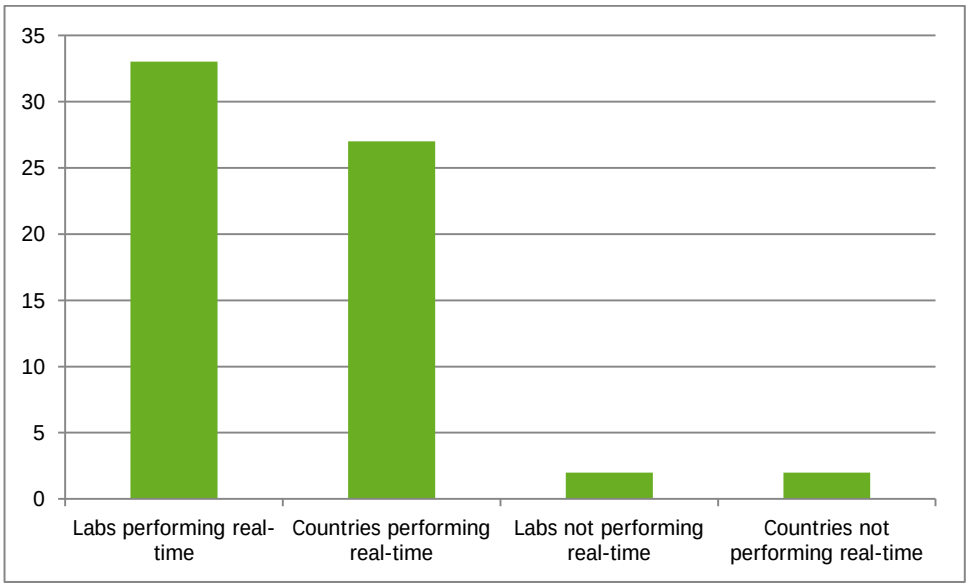
7.1 Responses

Prior to the virology task group's meeting in October 2011, responses to the questionnaire were received from 33 laboratories in 27 countries. Responses from the remaining two laboratories in two countries were received shortly after the meeting. Thus, responses were received from all laboratories and countries contacted. The responses were collated, and an analysis of the predicted ability of network laboratories to detect and subtype TRA H3N2 viruses with diagnostic RT-PCR assays was made.

7.2 Generic influenza A virus RT-PCR detection

Influenza A virus detection by RT-PCR is performed by all of the responding laboratories, with real-time RT-PCR performed by 33 (94%) laboratories in 27 countries in the network (Figure 1), and the remaining two laboratories in two countries performing generic influenza A detection by conventional RT-PCR.

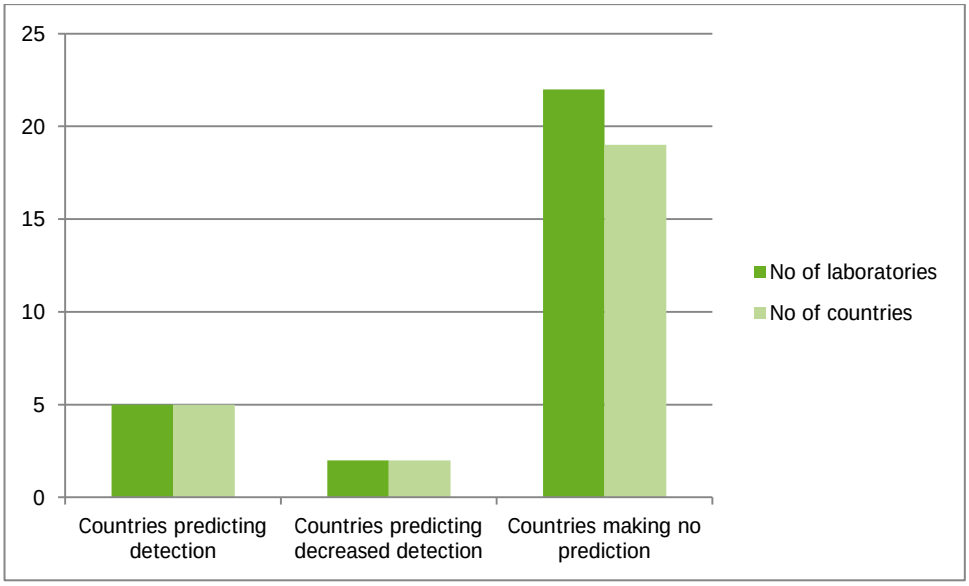
Figure 1.Generic influenza A detection by RT-PCR in the network



All laboratories performing generic influenza A detection, by either real-time or conventional RT-PCR use the matrix (M) gene as the generic influenza A assay target. Of the 33 laboratories performing real-time generic influenza A RT-PCR, 30 of the 33 provided the source or reference for the M-gene based real-time assay used, and the reference/method could be deduced from the information and/or primer/probe sequences provided by a further two laboratories. These 32 laboratories use 18 different generic influenza A real-time RT-PCR assays.

Based on the information provided by 24 laboratories in 20 countries, all predicted that the generic influenza A typing real-time RT-PCR assay employed in their laboratory would detect TRA H3N2 viruses (Figure 2). Only two of these laboratories (in two countries), predicted that sequence differences could lead to a reduction in detection sensitivity of their influenza A typing assays for the TRA H3N2 viruses represented in the exercise.

Figure 2. Predicted detection of TRA H3N2 viruses by generic influenza A M-gene real-time RT-PCR assays



Although five of the 29 laboratories in five countries did not make a prediction themselves on the ability of their generic influenza A typing real-time RT-PCR assay to detect TRA H3N2 viruses, the primer/probe sequence information provided allowed an assessment of this to be made for all five of these laboratories, either based on predictions made by other laboratories participating in the exercise who use the same primer/probe sets, or by comparison of their primer/probe sequences with the sequences provided. The generic influenza A real-time RT-PCR assays in use in these five laboratories would also be predicted to detect the TRA H3N2 viruses.

Four laboratories in four countries were unable to provide information on their generic influenza A RT-PCR primer and probe sequences, or their predicted ability to detect the TRA H3N2 M gene sequences, due to restrictions imposed on the sharing of primer/probe sequence information by the suppliers of the assays. However, the method referenced by two of these four laboratories was predicted to detect the TRA H3N2 viruses by another laboratory participating in the exercise that used the same assay.

Of the two laboratories in two countries which perform conventional generic influenza A RT-PCR targeting the M gene, one laboratory predicted that they would detect the TRA H3N2 viruses, and the other laboratory predicted a decrease in sensitivity of detection with their assay.

Real-time generic influenza A RT-PCR assays targeting the NP or NS genes were used in four network laboratories, but these laboratories also perform real-time influenza A M-gene typing RT-PCR assays.

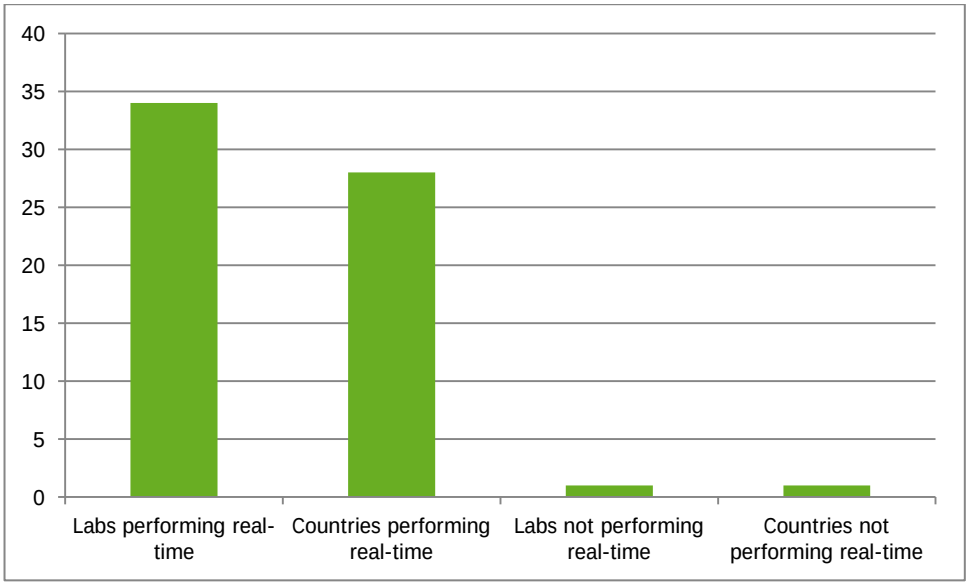
Three laboratories in three countries reported targeting the NP gene for detection, with two of the three predicting decreased detection of TRA H3N2 viruses, and one laboratory making no prediction on the capability of their assay to detect these viruses.

One laboratory reported the use of NS gene detection in real time RT-PCR and predicted that most, but not all, of the TRA H3N2 NS gene sequences included in the exercise would be detected.

7.3 Influenza A H3 RT-PCR subtyping

Real-time RT-PCR for detection of the influenza A(H3) subtype, usually performed by laboratories for seasonal H3N2 virus HA subtyping, is undertaken by 34 of the 35 responding CNRL network laboratories from 28 countries (Figure 3). The laboratory not performing real-time RT-PCR for H3 subtyping uses conventional RT-PCR for this purpose.

Figure 3. Influenza A H3 detection by real-time RT-PCR in the network



Of the 34 laboratories performing real-time H3 RT-PCR, 30 of the 34 provided the source or reference for the H3 real-time assay used, and the reference/method could be deduced from the information and/or primer/probe sequences provided by a further two laboratories. The 32 laboratories use 20 different H3 real-time RT-PCR assays.

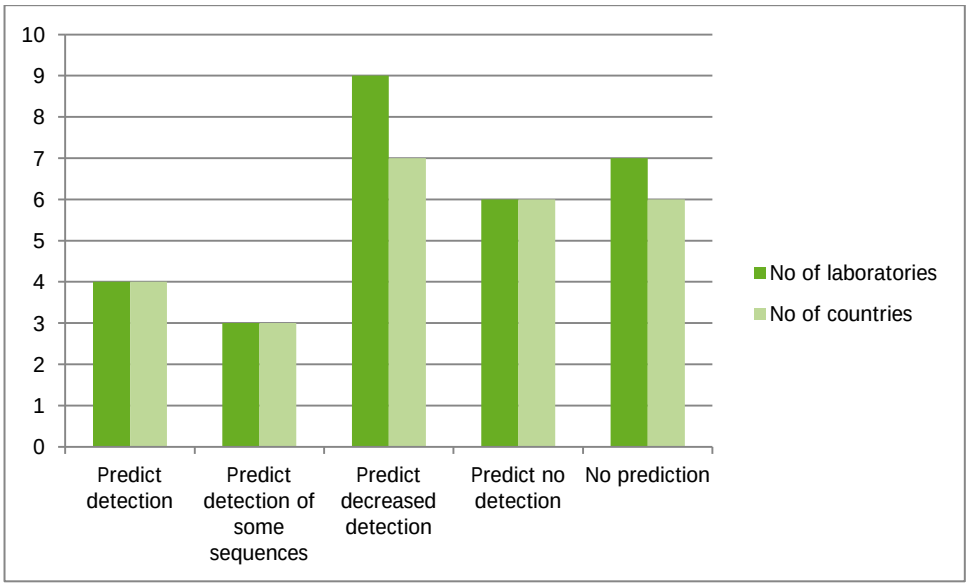
Information on real-time RT-PCR H3 specific primer and probe sequences was provided by 28 of the 34 laboratories in 23 countries. The remaining six laboratories were unable to provide information on their H3 subtyping RT-PCR primer and probe sequences, due to restrictions imposed on the availability and sharing of primer/probe sequence information by the suppliers of the assays. Four of these six laboratories were also unable to predict their ability to detect the TRA H3N2 HA gene sequences with their H3 assays, but the other two laboratories did make predictions. A total of 22 laboratories in 19 countries provided predictions on their capability for RT-PCR detection of the TRA H3N2 HA sequences (Figure 4).

Four laboratories in four countries predicted that their H3 assay would be capable of detecting the TRA H3N2 virus HA sequences provided, despite some sequence mismatches observed between the primers/probes and target regions.

Twelve laboratories in ten countries predicted either less sensitive detection of TRA H3N2 viruses with their H3 subtyping assays, or that not all the H3 virus HA sequences included in the exercise would be detected. The H3 subtyping assays used in the remaining six laboratories in six countries would not be expected to detect the HA of the TRA H3N2 viruses included in the exercise.

Seven laboratories in six countries made no predictions on the capability of their real-time H3 RT-PCR assays to detect the TRA H3N2 HA sequences. However, an assessment of this could be made based on either the predictions made by other laboratories participating in the exercise that use the same primer/probe sets, or by comparison of the primer/probe sequence information from the seven laboratories with the sequences provided in the exercise. The H3 assays in six of the seven laboratories in five countries would be predicted to have either less sensitive detection of TRA H3N2 viruses, or lack of detection of some of the H3 virus HA sequences included in the exercise. The H3 subtyping assay used in the remaining laboratory would be predicted to detect the HA of the TRA H3N2 viruses.

Figure 4. Predicted detection TRA H3N2 viruses by H3 subtyping real-time RT-PCR assays

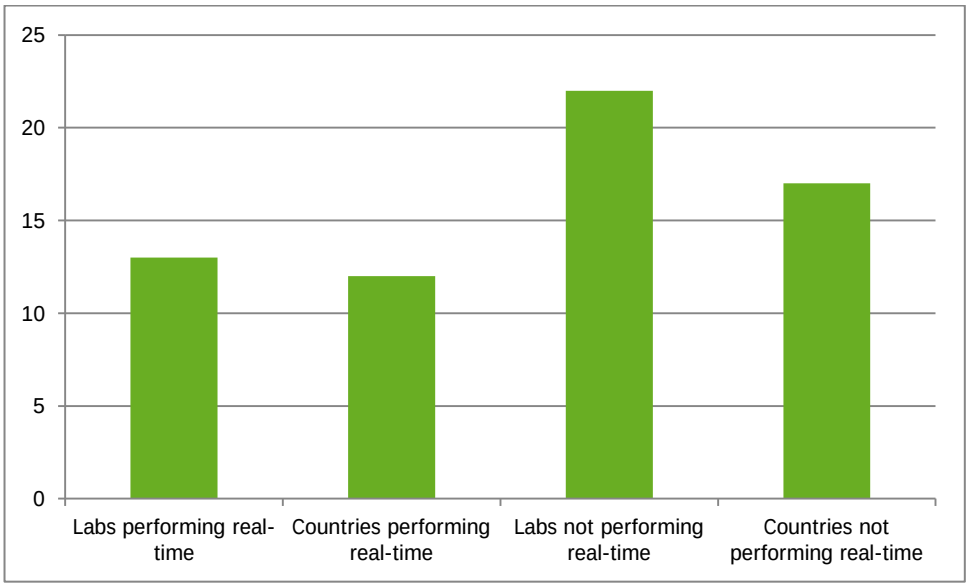


The one laboratory which performs conventional PCR for H3 subtyping, predicted that the assay would be less sensitive for detection of the TRA H3N2 HA sequences.

7.4 Influenza A N2 RT-PCR subtyping

For influenza A(N2) subtyping, 13 of the 35 laboratories in 12 countries stated that they perform real-time RT-PCR for this gene (Figure 5).

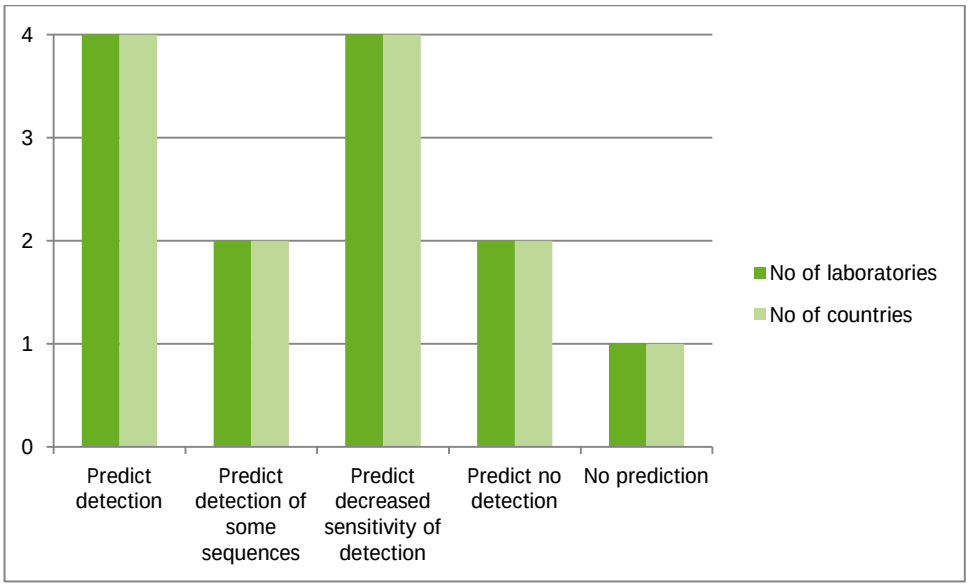
Figure 5. Influenza A(N2) detection by real-time RT-PCR in the network



Of these 13 laboratories, 12 provided a reference or method for the N2 real-time RT-PCR assay used, with 11 different methods being applied.

A prediction on the capability of the real-time RT-PCR N2 assays to detect the eight TRA H3N2 NA sequences provided, was made by 12 of the 13 laboratories (Figure 6).

Figure 6. **Predicted detection TRA H3N2 viruses by N2 subtyping real-time RT-PCR assays**



Four of the 13 laboratories in four countries predicted that they would be able to detect the TRA H3N2 NA sequences with their real-time RT-PCR N2 assays. Four laboratories in four countries predicted that they would detect the NA sequences, but that detection was expected to be less sensitive. Two laboratories predicted that some of the TRA H3N2 sequences would be detected, two laboratories predicted no detection and the remaining laboratory made no prediction on its ability to detect the NA sequences provided in the exercise.

Four of the 22 laboratories not performing real-time RT-PCR for N2 subtyping, in four countries, used conventional RT-PCR for this purpose. Only one of these laboratories made a prediction on the ability of the N2 assay to detect the TRA H3N2 NA sequences provided, with the PCR not expected to amplify four of the eight sequences and predicted to be suboptimal for amplification of two of the eight sequences. Primer sequences provided by two laboratories show a mismatch with some of the sequences provided, indicating that detection of some of the NA sequences would be predicted to be suboptimal.

8 Discussion and conclusions

The *in silico* exercise, which comprised the use of a questionnaire and alignments of representative TRA H3N2 swine virus gene sequences, was found to be a useful mechanism for the collection of technical information from, and predictions by CNRL network laboratories of their capability to detect the sequences by diagnostic typing and subtyping RT-PCR assays. The usability of the questionnaire would be better for both respondents, and for the rapid collation, analysis and interpretation of the responses, if it was web-based and used a series of 'drop down' menus for responses where possible.

There was excellent participation by the network in the *in silico* exercise; responses were returned by all laboratories and countries that received the questionnaire and alignments. Influenza A virus detection by real-time RT-PCR is performed by the majority of responding laboratories and countries. Where predictions could be made, all laboratories expected that the generic influenza A typing real-time RT-PCR assay employed in their laboratory would be capable of detecting TRA H3N2 viruses. Only two of these laboratories (in two countries), predicted that sequence differences could lead to a reduction in detection sensitivity for the TRA H3N2 viruses represented in the exercise. Further comparison of the primer/probe and target sequence mismatches indicates that in one of these two laboratories, sensitivity of detection would not be affected, and in the other laboratory the generic influenza A assay would be predicted to be less sensitive for only one of the TRA H3N2 M-gene sequences provided.

Further human infections with TRA H3N2 viruses in the United States in 2011 have been reported subsequent to the distribution of the *in silico* exercise. The viral gene sequences from these recent cases are similar to those included in the CNRL exercise, but have through reassortment acquired an M gene derived from the A(H1N1)pdm09 virus [13-17]. As part of the pandemic response in 2009, CNRL laboratories have already established the capability to detect A(H1N1)pdm09 viruses by generic influenza A RT-PCR. Since all responding laboratories use the M gene as the generic influenza A target in either real-time (33 laboratories in 27 countries) or conventional (two laboratories in two countries) diagnostic RT-PCR assays, they would be predicted to detect the M gene of TRA H3N2 viruses from the most recent cases in the United States.

The analysis of the responses to the *in silico* exercise therefore indicates that the network has a very good capability for detection of TRA H3N2 viruses by generic influenza A real-time RT-PCR assays, with all laboratories predicting that their generic real-time influenza A assays would detect the TRA H3N2 viruses represented in the M-gene alignment.

In contrast, the *in silico* exercise highlighted that there are gaps in the CNRL network for HA and NA subtype RT-PCR determination of North American TRA H3N2 swine origin viruses. Real-time RT-PCR for detection of the influenza A(H3) subtype is undertaken by most of the responding CNRL laboratories and countries. Where predictions were made by laboratories on the ability of H3 assays to detect TRA H3N2 viruses, 16 laboratories in 13 countries predicted that the H3 real-time assays would detect these viruses, but 12 laboratories in 10 countries that made predictions stated that they would expect reduced sensitivity in detection for some, or all of the HA sequences in the exercise. Moreover, six laboratories in six countries that made predictions, expected no H3 subtyping detection of the TRA H3N2 viruses based on the HA sequences provided. Real-time RT-PCR for detection of the influenza A(N2) subtype is performed by fewer laboratories. Detection of the N2 subtype is useful as a confirmatory tool where equivocal or unclear results are obtained for generic influenza A and/or H3 subtype detection, and can be used to monitor reassortment events where acquisition of a different NA gene could lead to the generation of a virus with a novel HA/NA subtype combination; e.g. the acquisition of the H1 gene from seasonal H1N1 virus by seasonal H3N2 virus in 2001 [18]. Where a prediction was made by laboratories on the ability of real-time N2 assays to detect TRA H3N2 viruses, ten laboratories in nine countries expected to detect TRA H3N2 viruses however six laboratories in six countries predicted reduced sensitivity for some or all of the NA sequences provided, and two laboratories in two countries expected no detection of TRA H3N2 NA. Details on primer and probe sequences, predictions of ability to detect the provided sequences, and/or the reference for the diagnostic real-time typing and subtyping assays were provided by the majority of respondents. Where primer and probe sequences were provided by laboratories but no prediction of ability to detect the provided gene sequences was made, prediction was performed by collators. However, primer/probe sequences were not provided by some laboratories due to non-disclosure agreements with the assay supplier(s). In addition to the information provided, where countries have more than one CNRL laboratory, there is a requirement to better understand how the capability of these laboratories relates to detection and identification of TRA H3N2 viruses in their country.

There is also a need for a review of algorithms for influenza virus detection and identification used in CNRL laboratories. The analysis of the responses to the *in silico* exercise indicated that the network has a very good capability for detecting TRA H3N2 viruses by generic influenza A real-time RT-PCR assays, and less capability for detection and origin identification of H3 or N2 with existing subtyping assays. Therefore, the algorithms used in laboratories will determine if TRA H3N2 viruses are firstly detected, then subtyped as H3N2 and lastly identified as of swine origin. If a generic influenza A assay is used for diagnosis, then TRA H3N2 viruses should be detected by the majority of laboratories. However, if samples are subtyped directly without influenza A screening first, then

TRA H3N2 viruses might be missed as there are gaps in TRA H3N2 HA subtyping in the network. It is also important to consider the subsequent characterisation of TRA H3N2 viruses detected by generic influenza A assays, and distinguishing TRA H3N2 viruses from seasonal H3N2 viruses.

Suspected TRA H3N2 viruses identified as influenza A positive, but H3 negative and unsubtypable for seasonal H1N1 and (H1N1)pdm09, should be sent without delay to the World Health Organization Collaborating Centre (WHO CC) for further analysis. Similarly, viruses identified as influenza A positive, but with unusually high H3 RT-PCR Ct values (i.e. an imbalance between low M gene Ct value and high H3 Ct value) should also trigger further investigation by laboratories. However, in most laboratories, only a proportion of viruses that are identified as influenza A H3 subtype (i.e. balanced M and H3 Ct values) are further characterised genetically and antigenically, so swine origin H3N2 viruses could be missed. It is important to detect and characterise TRA H3N2 viruses as rapidly as possible, as early detection and analysis of the properties of these viruses will enable rapid risk assessment of the threat to human health. Any human infections with swine TRA H3N2 viruses that have been laboratory confirmed should be included in The European Surveillance System (TESSy) reporting, with a comment given in the comment field, and isolates should be sent as a matter of urgency to the Mill Hill WHO Collaborating Centre for detailed analysis. Furthermore, human infections with novel influenza viruses are notifiable through the International Health Regulations.

It should be noted that a limitation of the assessment was that the *in silico* exercise focused on detection of TRA H3N2 North American viruses, hence the gene alignments did not contain examples of European or Asian swine viruses. Since there was a high confidence in the ability of M gene PCR assays to detect TRA H3N2 swine viruses across the network, but lower reliability for PCR-based subtyping, M gene positive samples that are subtyped not determined should be rapidly investigated using alternative approaches to help overcome this problem.

The conclusions of the in silico exercise are based on the predictions made by network laboratories, following comparison of primer/probe sequences with target regions in the sequence alignments provided. The accuracy of the predictions on the capability of detection by typing and subtyping diagnostic assays relies upon the experience and judgement of the responding laboratories and data collators. Furthermore, not all respondents made predictions, or included the reference for assays used, and some were unable to include information on their primer/probes due to material transfer agreement (MTA) restrictions imposed by assay suppliers. The interpretation of the responses to the questionnaire therefore also relies upon the accuracy and extent of the information provided. The capability for detection and subtyping of TRA H3N2 viruses determined from this theoretical exercise should therefore be assessed in vitro, by the use of a proficiency panel. The panel should ideally;

- contain representative North American TRA H3N2 swine origin viruses, including recent strains which have an M gene derived from (H1N1)pdm09 viruses.
- be extended to contain examples of representative Eurasian swine viruses.
- include some of the TRA H3N2 viruses included in the *in silico* exercise, to allow assessment of the predictions made.

The use of a proficiency panel will provide an assessment of the validity of the findings of the *in silico* exercise, and inform on the usefulness of such an approach for future preparedness and planning in the network. The use of a panel together with understanding the algorithms in use in the network, will advise on which assays have the capability for detection, subtyping and origin identification of swine influenza viruses, and aid laboratories and countries in understanding their capability for both detection of TRA H3N2 viruses and their distinguishing from seasonal H3N2 viruses.

9 Recommendations

- Distribute a proficiency panel to network laboratories and formally correlate practical testing results with predictions from the *in silico* exercise
- Aggregate information on the application of test algorithms in countries
- Prepare to make available standard operating procedures (SOPs) which improve the detection of swine H3 HA
- Move towards greater harmonisation of methodology employed for HA detection by RT-PCR
- Improve the ability of laboratories to link primer/probe sequences with the ability to predict detection, through the use of proficiency panel testing and through continuing inclusion and improvement of bioinformatics analysis in training programs

10 References

1. Scholtissek, C., Burger, H., Kistner, O. and Shortridge,KF. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology. 1985; 147: 287–294.
2. Ma, W., Lager, KM., Vincent, AL., Janke, BH., Gramer, MR., Richt, JA. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health Review. 2009: 56(6–7):326–37.
3. Brown, IH., The epidemiology and evolution of influenza viruses in pigs. Veterinary Microbiology. 2000: 74, 29–46.
4. Koen, JS. A practical method for field diagnosis of swine diseases. Journal of the American Veterinary Medical Association. . 1919: 14, 468–470.
5. Shope, RE. Swine Influenza. Filtration experiments and etiology. Journal of Experimental Medicine. 1931: 54, 373–385.
6. Easterday, BC., Van Reeth, K.: Swine Influenza. In 'Diseases of Swine' (BE Straw, S D'Allaire, WL Mengeling, DJ Taylor, Eds), Iowa State University Press, Ames 8th Ed. 1999: 277–290.
7. Richt, J., Larger, KM., Jeanke, BH., Woods, RD., Webster, RG., Webby RJ.: Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 SIV co-circulating in the United States. Journal of Clinical Microbiology. 2003:41, 3198–3205.
8. Choi, YK., Goyal, SM., Farnham, MW., Joo, HS. Phylogenic analysis of H1N2 isolates of influenza A virus from pigs in the United States. Virus Research. 2002: 87, 173–179.
9. Webby, RJ., Rossow, K., Erickson, G., Sims, Y., Webster, R. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Research. 2004: 103(1–2), 67–73.
10. Myers KP., Olsen, CW., Gray, GC. Cases of swine influenza in humans: a review of the literature. Clinical Infectious Diseases. 2007: 44(8), 1084–1088.
11. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/flu/weekly/weeklyarchives2010–2011/weekly44.htm.
12. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/flu/swineflu/variant-cases-us.htm.
13. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6035a6.htm?s_cid=mm6035a6_w.
14. Centers for Disease Control and prevention. 2011: Available at; http://www.cdc.gov/media/haveyouheard/stories/lab_testing.html
15. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6035a6.htm?s_cid=mm6035a6_w
16. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/flu/weekly/
17. Centers for Disease Control and Prevention. 2011: Available at; http://www.cdc.gov/flu/swineflu/soiv_cases.htm
18. Gregory, V., Bennett, M., Orkhan, MH., Al Hajjar, S., Varsano, N., Mendelson, E., Zambon, M., Ellis, J., Hay, A., Lin, YP. Emergence of influenza A H1N2 reassortant viruses in the human population during 2001. Virology. 2002: Aug 15;300(1):1–7.

Appendix 1. Questionnaire

Name of laboratory and person completing questionnaire:

Email address:

Please answer the questions below concerning RT-PCR assays in your lab for detection of influenza AH3N2 viruses:

		A/MP	A/NP	A/NS	A/H3	A/N2
A)	**Real time RT-PCR**					
i)	Which genes are targeted in real-time RT-PCR assays in your laboratory for typing and subtyping influenza AH3N2 viruses – Enter Yes or No in appropriate columns					
ii)	Position of forward primer(s) & sequence. Enter position of 5' nucleotide using numbering in alignment, followed by the sequence of forward primer					
iii)	Position of reverse primer(s) & sequence. Enter position of 5' nucleotide using numbering in alignment, followed by the sequence of the reverse primer					
iv)	Position of probe(s) and sequence. Enter position of 5' nucleotide using numbering in alignment, followed by the probe sequence					
v)	Predicted detection of viruses in alignment. Please describe position of any mismatches in primers/probes versus which target sequences and if possible predict the ability of your assay(s) to detect the viruses in the alignment, based on sequence homology between primers/probes and the target sequences					
vi)	Reference (publication/SOP) for assay(s)					
vii)	Other comments/information					
B)	**Conventional RT-PCR**					
i)	Which genes are targeted in conventional RT-PCR assays in your laboratory for typing and subtyping influenza AH3N2 viruses – Enter Yes or No in appropriate columns					
ii)	Position of forward primer(s) & sequence. Enter position of 5' nucleotide using numbering in alignment, followed by the forward primer sequence					
iii)	Position of reverse primer(s) & sequence. Enter position of 5' nucleotide using numbering in alignment, followed by the sequence of the reverse primer					
iv)	Predicted detection of viruses in alignment. Please describe position of any mismatches in primers versus which target sequences and if possible predict the ability of your assay(s) to detect the viruses in the alignment, based on sequence homology between primers/probes and the target audience					
v)	Reference (publication/SOP) for assay(s)					
vi)	Other comments/information					

Appendix 2. List of responding laboratories by country

Country	Institution
Austria	AKH Wien - Medical University Vienna, Vienna
Belgium	Scientific Institute of Public Health, Brussels
Bulgaria	National Center of Infectious and Parasitic Diseases, Sofia
Cyprus	Nicosia General Hospital Microbiology Laboratory, Nicosia
Czech Republic	National Institute of Public Health, Prague
Denmark	Statens Serum Institute, Copenhagen
Estonia	Laboratory of Communicable Diseases, Tallinn
Finland	National Institute for Health and Welfare, Helsinki
France	Lyon Centre Hospitalier Lyon Sud, Lyon.
France	CNR de la Grippe - Institute Pasteur, Paris
Germany	Robert Koch Institute, Berlin
Greece	National Influenza Centre for Southern Greece, Hellenic Pasteur Institute, Athens
Greece	National Influenza Centre for Northern Greece, Thessaloniki
Hungary	National Center for Epidemiology, Budapest
Iceland	National University Hospital, Reykjavik
Ireland	University College Dublin, Dublin
Italy	Istituto Superiore di Sanita (NIH), Rome
Latvia	Infectology Center of Latvia
Lithuania	National Public Health Surveillance Laboratory, Vilnius
Luxembourg	National Health Laboratory, Luxembourg
Malta	Mater Dei Hospital, Malta
Netherlands	National Institute of Public Health and the Environment (RIVM), Bilthoven
Norway	Norwegian Institute of Public Health, Oslo
Poland	National Institute of Public Health - National Institute of Hygiene, Warsaw
Portugal	National Institute of Health, Lisboa
Romania	Cantacuzino Institute, Bucharest
Slovakia	Public Health Authority of Slovak Republic, Bratislava
Slovenia	National Institute of Public Health, Ljubljana
Spain	National Influenza Centre, Barcelona
Spain	National Influenza Centre, Valladolid
Sweden	Swedish Institute for Infectious Disease Control, Solna
United Kingdom	West of Scotland Specialist Virology Centre, Glasgow, Scotland
United Kingdom	Health Protection Agency - Microbiology Services, London, England
United Kingdom	Regional Virus laboratory, Belfast, Northern Ireland
United Kingdom	Specialist Virology Centre for Wales, Cardiff, Wales

Appendix 3. Gene alignments included in the *in silico* exercise

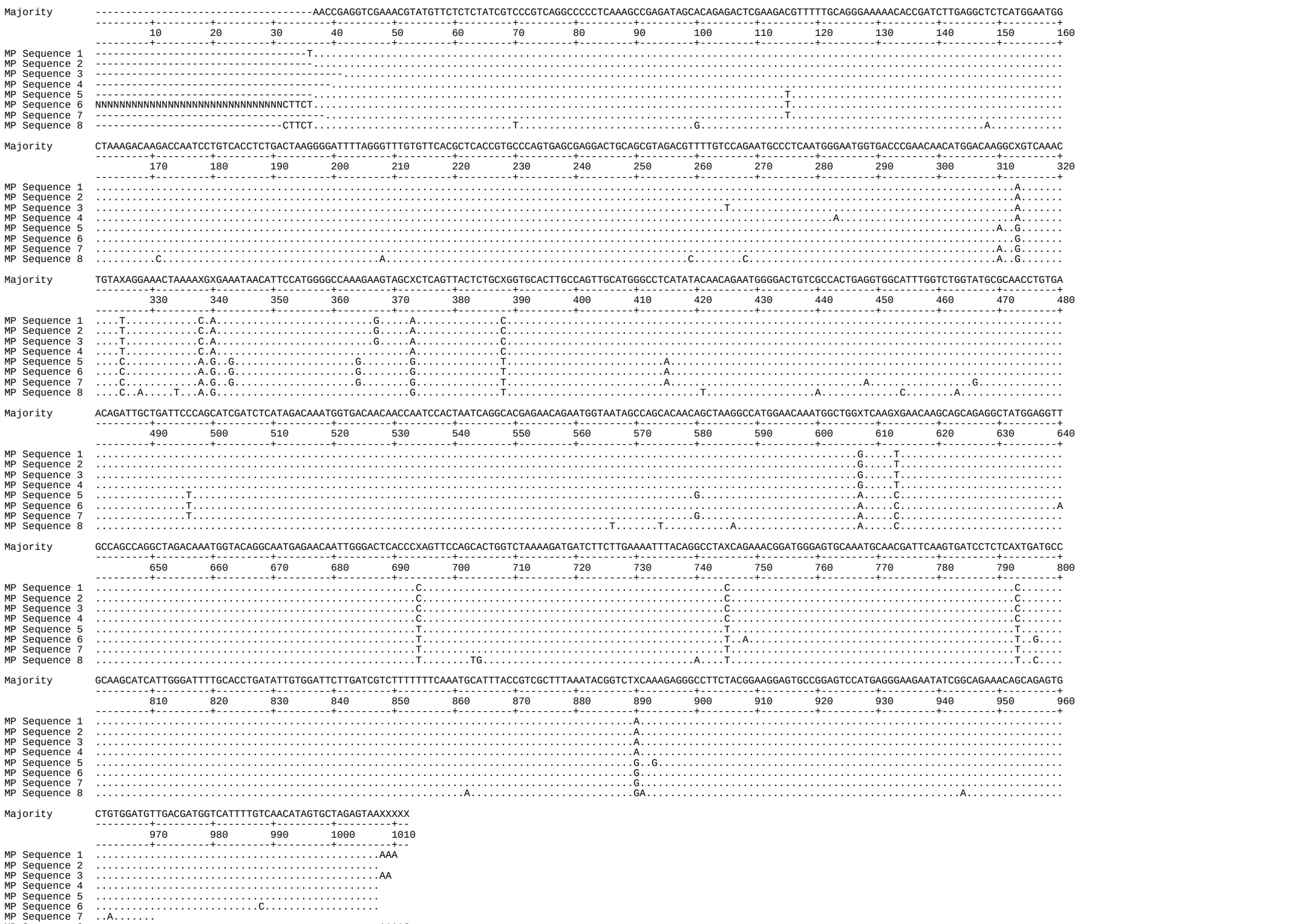
Alignments of representative nucleotide sequences for the matrix (MP), non-structural (NS), nucleoprotein (NP), haemagglutinin (HA) and neuraminidase (NA) genes of eight North American TRA H3N2 swine origin viruses associated with human or swine infections, as available in early 2011, are shown.

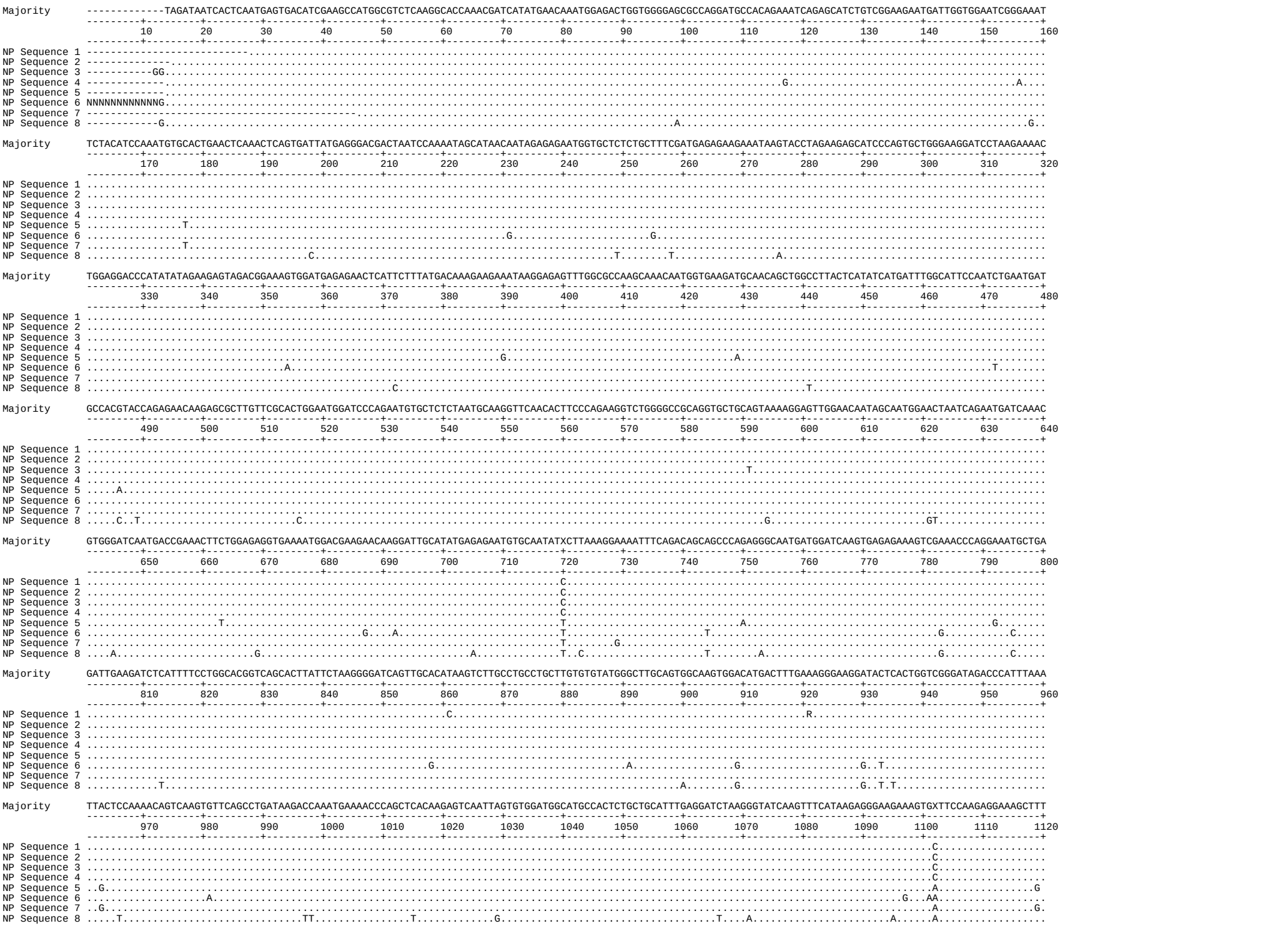
The majority sequence for each gene determined from comparison of the eight sequences is displayed above the aligned virus gene sequences. An 'x' denotes where the majority nucleotide sequence cannot be determined.

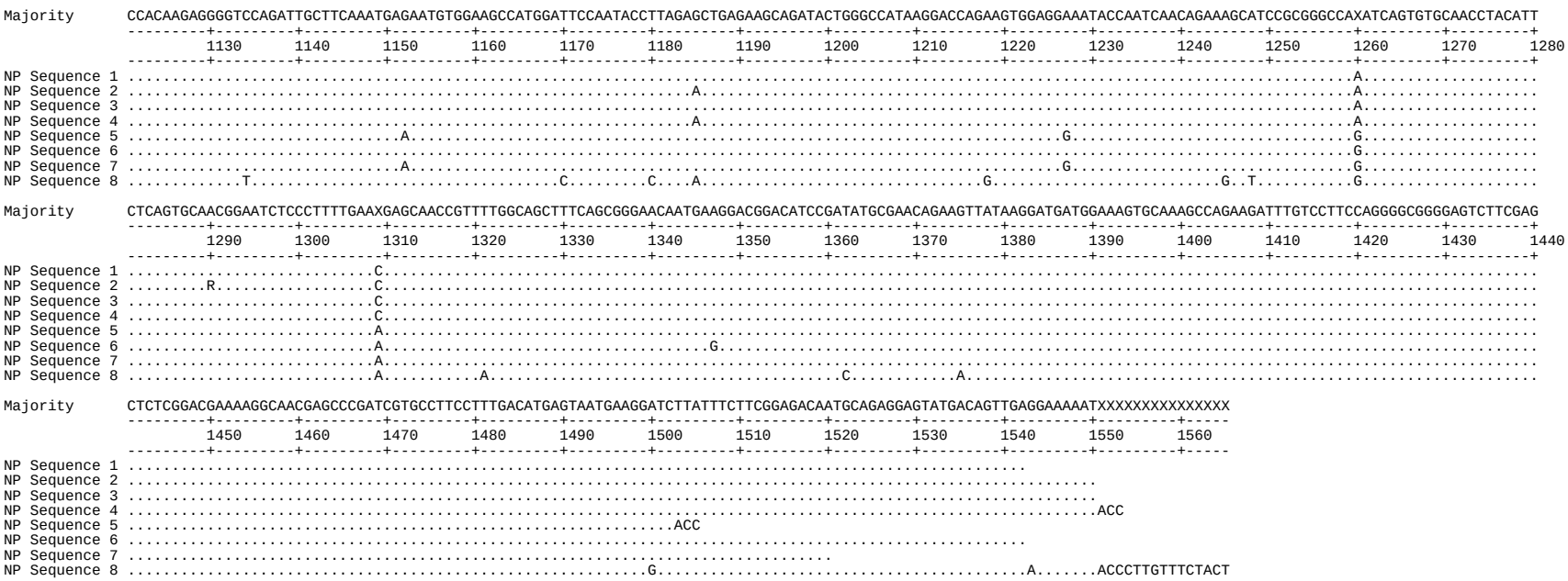
Nucleotide differences in individual sequences compared to the majority are displayed, with nucleotides identical to the majority sequence shown as a dot (.)

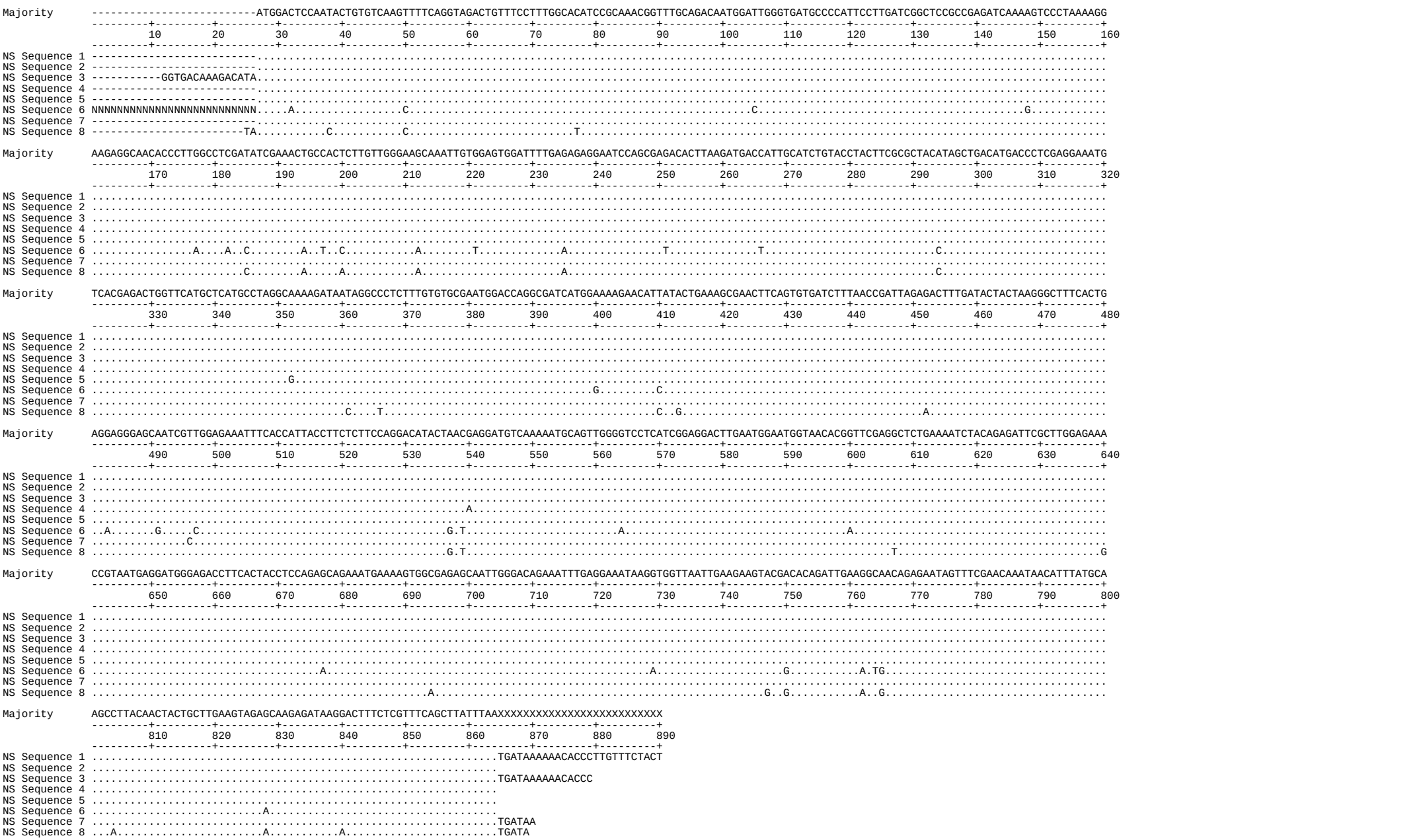
A dash (-) indicates the nucleotide at this position was not present in the available sequence.

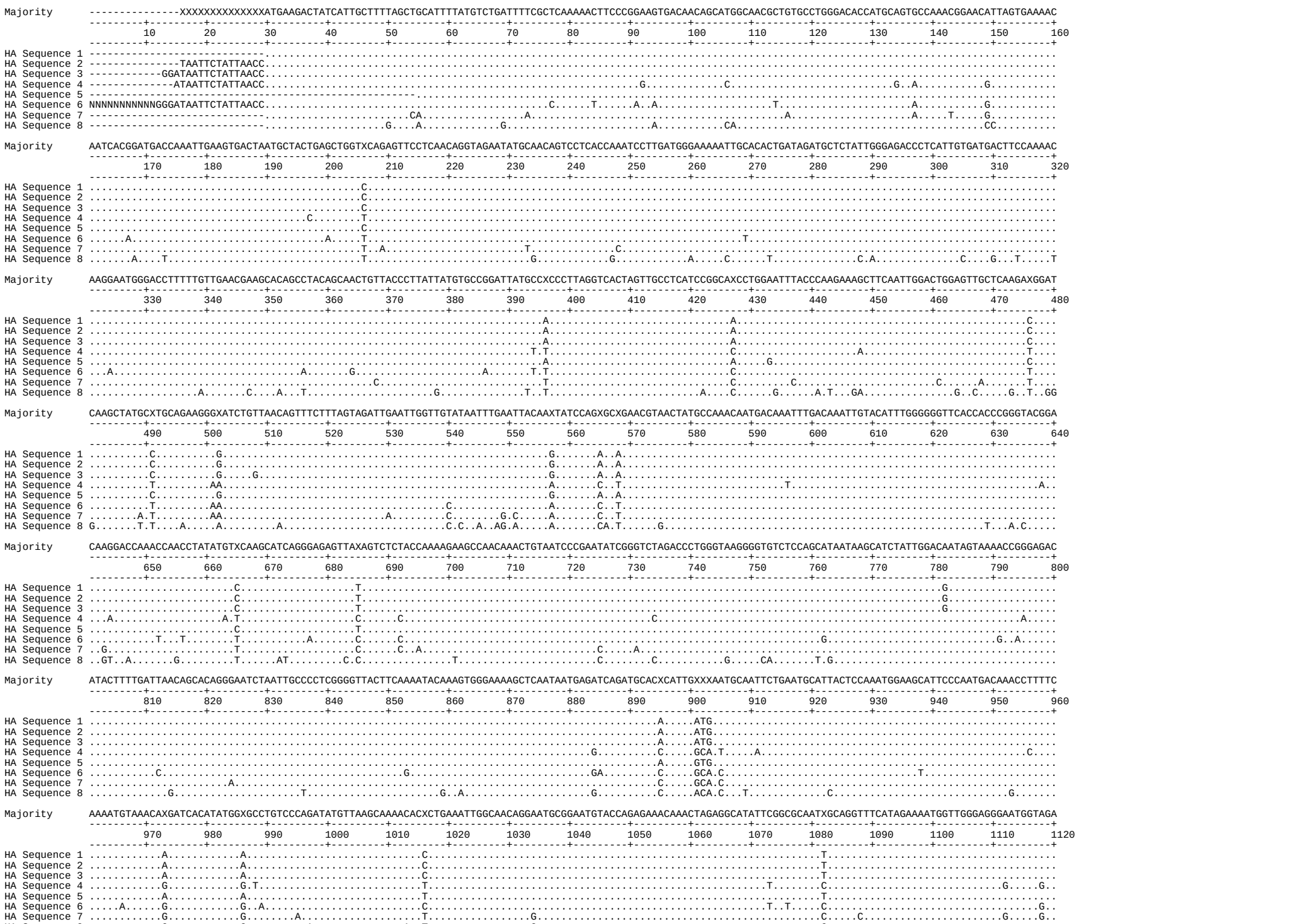
Laboratories based the predicted detection of TRA H3N2 viruses represented in the sequence alignments following comparison of RT-PCR assay primer/probe sequences, with their target regions.

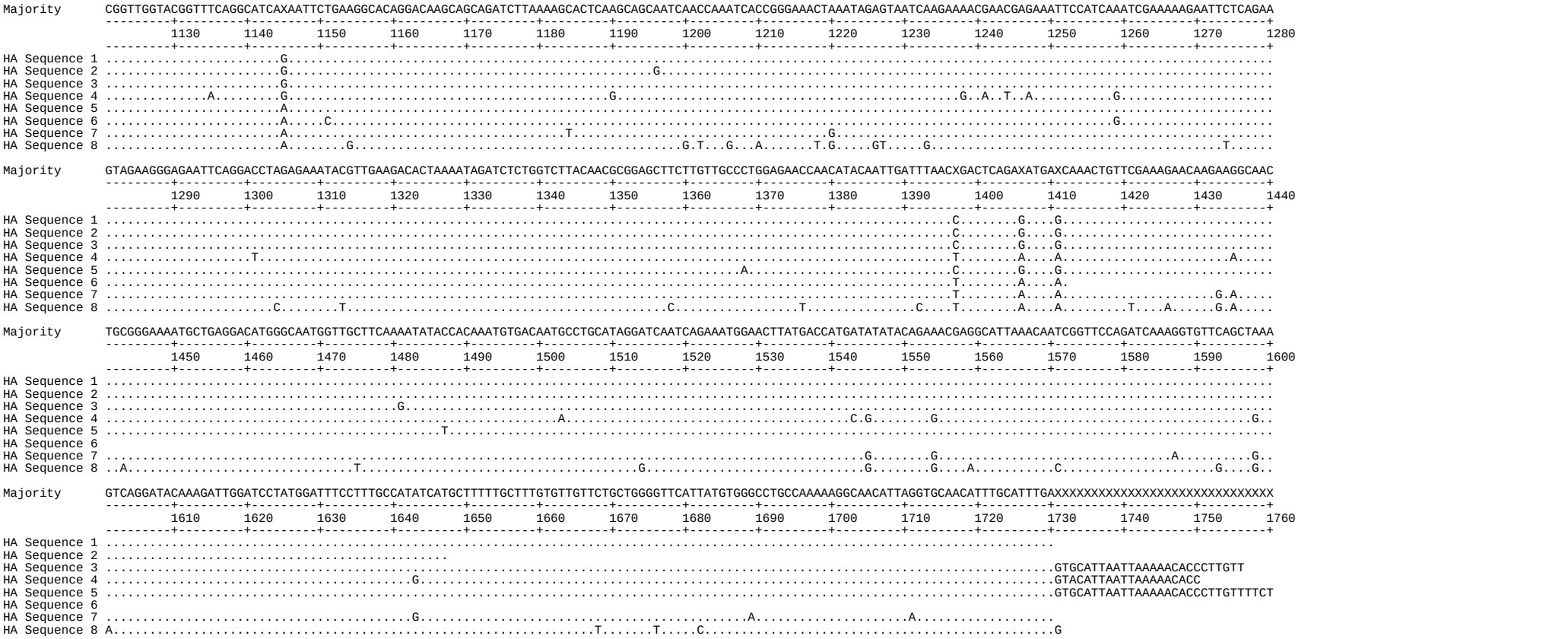


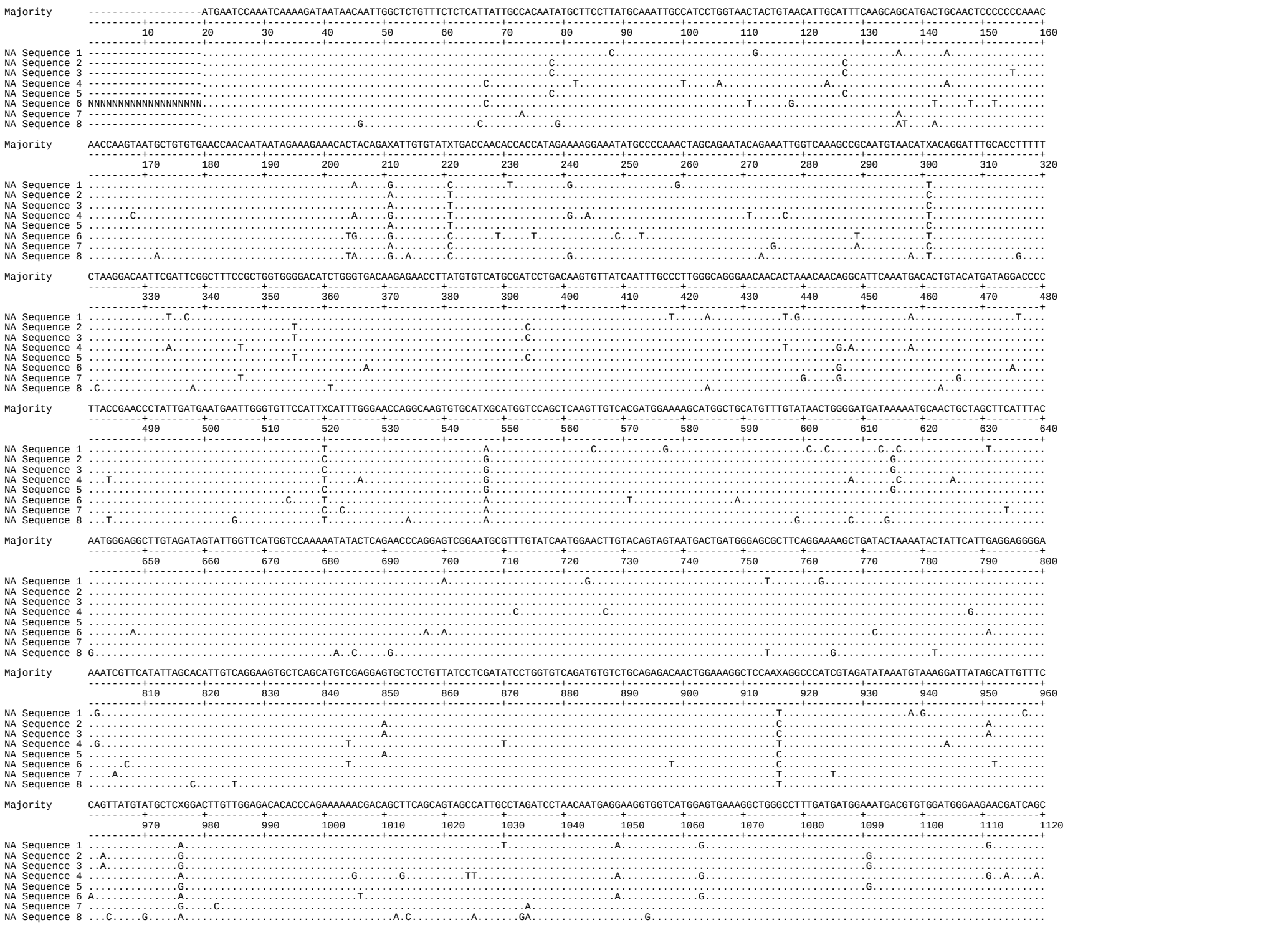

Majority TAGATAATCACTCAATGAGTGACATCGAAGCCATGGCGTCTCAAGGCACCAAACGATCATATGAACAAATGGAGACTGGTGGGGAGCGCCAGGATGCCACAGAAATCAGAGCATCTGTCGGAAGAATGATTGGTGGAATCGGGAAAT
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
NP Sequence 1
NP Sequence 2
NP Sequence 3 GG
NP Sequence 4 G A
NP Sequence 5
NP Sequence 6 NNNNNNNNNNNG
NP Sequence 7
NP Sequence 8 G A G
Majority TCTACATCCAAATGTGCACTGAACTCAAACTCAGTGATTATGAGGGACGACTAATCCAAAATAGCATAACAATAGAGAGAATGGTGCTCTCTGCTTTCGATGAGAGAAGAAATAAGTACCTAGAAGAGCATCCCAGTGCTGGGAAGGATCCTAAGAAAAC
170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320
NP Sequence 1
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5 T
NP Sequence 6 G G
NP Sequence 7 T
NP Sequence 8 C T T A
Majority TGGAGGACCCATATATAGAAGAGTAGACGGAAAGTGGATGAGAGAACTCATTCTTTATGACAAAGAAGAAATAAGGAGAGTTTGGCGCCAAGCAAACAATGGTGAAGATGCAACAGCTGGCCTTACTCATATCATGATTTGGCATTCCAATCTGAATGAT
330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480
NP Sequence 1
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5 G A
NP Sequence 6 A T
NP Sequence 7
NP Sequence 8 C T
Majority GCCACGTACCAGAGAACAAGAGCGCTTGTTCGCACTGGAATGGATCCCAGAATGTGCTCTCTAATGCAAGGTTCAACACTTCCCAGAAGGTCTGGGGCCGCAGGTGCTGCAGTAAAAGGAGTTGGAACAATAGCAATGGAACTAATCAGAATGATCAAAC
490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640
NP Sequence 1
NP Sequence 2
NP Sequence 3 T
NP Sequence 4
NP Sequence 5 A
NP Sequence 6
NP Sequence 7
NP Sequence 8 C T G GT
Majority GTGGGATCAATGACCGAAACTTCTGGAGAGGTGAAAATGGACGAAGAACAAGGATTGCATATGAGAGAATGTGCAATATXCTTAAAGGAAAATTTCAGACAGCAGCCCAGAGGGCAATGATGGATCAAGTGAGAGAAAGTCGAAACCCAGGAAATGCTGA
650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800
NP Sequence 1 C
NP Sequence 2 C
NP Sequence 3 C
NP Sequence 4 C
NP Sequence 5 T T A G
NP Sequence 6 G A T T G C
NP Sequence 7 T G
NP Sequence 8 A G A T C T A G C
Majority GATTGAAGATCTCATTTTCCTGGCACGGTCAGCACTTATTCTAAGGGGATCAGTTGCACATAAGTCTTGCCTGCCTGCTTGTGTGTATGGGCTTGCAGTGGCAAGTGGACATGACTTTGAAAGGGAAGGATACTCACTGGTCGGGATAGACCCATTTAAA
810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960
NP Sequence 1 C R
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5
NP Sequence 6 G A G G T
NP Sequence 7
NP Sequence 8 T A G G T T
Majority TTACTCCAAAACAGTCAAGTGTTCAGCCTGATAAGACCAAATGAAAACCCAGCTCACAAGAGTCAATTAGTGTGGATGGCATGCCACTCTGCTGCATTTGAGGATCTAAGGGTATCAAGTTTCATAAGAGGGAAGAAAGTGXTTCCAAGAGGAAAGCTTT
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100 1110 1120
NP Sequence 1 C
NP Sequence 2 C
NP Sequence 3 C
NP Sequence 4 C
NP Sequence 5 G A G
NP Sequence 6 A G AA
NP Sequence 7 G A G
NP Sequence 8 T TT T G T A A A A A

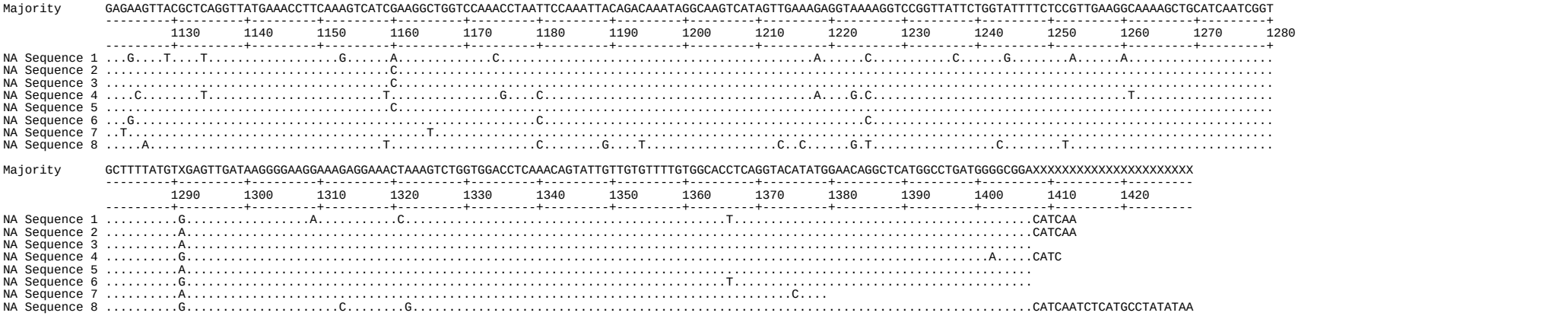

Majority CCACAAGAGGGGTCCAGATTGCTTCAAATGAGAATGTGGAAGCCATGGATTCCAATACCTTAGAGCTGAGAAGCAGATACTGGGCCATAAGGACCAGAAGTGGAGGAAATACCAATCAACAGAAAGCATCCGCGGGCCAXATCAGTGTGCAACCTACATT
1130 1140 1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280
NP Sequence 1
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5
NP Sequence 6
NP Sequence 7
NP Sequence 8
Majority CTCAGTGCAACGGAATCTCCCTTTTGAAXGAGCAACCGTTTTGGCAGCTTTCAGCGGGAACAATGAAGGACGGACATCCGATATGCGAACAGAAGTTATAAGGATGATGGAAAGTGCAAAGCCAGAAGATTTGTCCTTCCAGGGGCGGGGAGTCTTCGAG
1290 1300 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
NP Sequence 1
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5
NP Sequence 6
NP Sequence 7
NP Sequence 8
Majority CTCTCGGACGAAAAGGCAACGAGCCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAATGCAGAGGAGTATGACAGTTGAGGAAAAATXXXXXXXXXXXXXXX
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
NP Sequence 1
NP Sequence 2
NP Sequence 3
NP Sequence 4
NP Sequence 5
NP Sequence 6
NP Sequence 7
NP Sequence 8


Majority ----------ATGGACTCCAATACTGTGTCAAGTTTTCAGGTAGACTGTTTCCTTTGGCACATCCGCAAACGGTTTGCAGACAATGGATTGGGTGATGCCCCATTCCTTGATCGGCTCCGCCGAGATCAAAAGTCCCTAAAAGG
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
NS Sequence 1 ----------.....
NS Sequence 2 ----------.....
NS Sequence 3 ----------GGTGACAAAGACATA.....
NS Sequence 4 ----------.....
NS Sequence 5 ----------.....
NS Sequence 6 NNNNNNNNNNNNNNNNNNNNNNNNN.....A.....C.....C.....G.....
NS Sequence 7 ----------.....
NS Sequence 8 ----------TA.....C.....C.....T.....
Majority AAGAGGCAACACCCTTGGCCTCGATATCGAAACTGCCACTCTTGTTGGGAAGCAAATTGTGGAGTGGATTTTGAGAGAGGAATCCAGCGAGACACTTAAGATGACCATTGCATCTGTACCTACTTCGCGCTACATAGCTGACATGACCCTCGAGGAAATG
170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320
NS Sequence 1 .....
NS Sequence 2 .....
NS Sequence 3 .....
NS Sequence 4 .....
NS Sequence 5 .....
NS Sequence 6 .....A....A..C.....A..T..C.....A.....T.....A.....T.....T.....C.....
NS Sequence 7 .....
NS Sequence 8 .....C.....A....A.....A.....A.....C.....
Majority TCACGAGACTGGTTCATGCTCATGCCTAGGCAAAAGATAATAGGCCCTCTTTGTGTGCGAATGGACCAGGCGATCATGGAAAAGAACATTATACTGAAAGCGAACTTCAGTGTGATCTTTAACCGATTAGAGACTTTGATACTACTAAGGGCTTTCACTG
330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480
NS Sequence 1 .....
NS Sequence 2 .....
NS Sequence 3 .....
NS Sequence 4 .....
NS Sequence 5 .....G.....
NS Sequence 6 .....G.....C.....
NS Sequence 7 .....
NS Sequence 8 .....C...T.....C..G.....A.....
Majority AGGAGGGAGCAATCGTTGGAGAAATTTCACCATTACCTTCTCTTCCAGGACATACTAACGAGGATGTCAAAAATGCAGTTGGGGTCCTCATCGGAGGACTTGAATGGAATGGTAACACGGTTCGAGGCTCTGAAAATCTACAGAGATTCGCTTGGAGAAA
490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640
NS Sequence 1 .....
NS Sequence 2 .....
NS Sequence 3 .....
NS Sequence 4 .....A.....
NS Sequence 5 .....
NS Sequence 6 ..A.....G.....C.....G.T.....A.....A.....
NS Sequence 7 .....C.....
NS Sequence 8 .....G.T.....T.....G
Majority CCGTAATGAGGATGGGAGACCTTCACTACCTCCAGAGCAGAAATGAAAAGTGGCGAGAGCAATTGGGACAGAAATTTGAGGAAATAAGGTGGTTAATTGAAGAAGTACGACACAGATTGAAGGCAACAGAGAATAGTTTCGAACAAATAACATTTATGCA
650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800
NS Sequence 1 .....
NS Sequence 2 .....
NS Sequence 3 .....
NS Sequence 4 .....
NS Sequence 5 .....
NS Sequence 6 .....A.....A.....G.....A.TG.....
NS Sequence 7 .....
NS Sequence 8 .....A.....G..G.....A..G.....
Majority AGCCTTACAACTACTGCTTGAAGTAGAGCAAGAGATAAGGACTTTCTCGTTTCAGCTTATTTAAXXXXXXXXXXXXXXXXXXXXXXXXXXX
810 820 830 840 850 860 870 880 890
NS Sequence 1 .....TGATAAAAAACACCCTTGTTTCTACT
NS Sequence 2 .....
NS Sequence 3 .....TGATAAAAAACACCC
NS Sequence 4 .....
NS Sequence 5 .....
NS Sequence 6 .....A.....
NS Sequence 7 .....TGATAA
NS Sequence 8 ...A.....A.....A.....TGATA


Majority
HA Sequence 1
HA Sequence 2
HA Sequence 3
HA Sequence 4
HA Sequence 5
HA Sequence 6
HA Sequence 7
HA Sequence 8


Majority CGGTTGGTACGGTTTCAGGCATCAXAATTCTGAAGGCACAGGACAAGCAGCAGATCTTAAAAGCACTCAAGCAGCAATCAACCAAATCACCGGGAAACTAAATAGAGTAATCAAGAAAACGAACGAGAAATTCCATCAAATCGAAAAAGAATTCTCAGAA
1130 1140 1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280
HA Sequence 1
HA Sequence 2
HA Sequence 3
HA Sequence 4
HA Sequence 5
HA Sequence 6
HA Sequence 7
HA Sequence 8
Majority GTAGAAGGGAGAATTCAGGACCTAGAGAAATACGTTGAAGACACTAAAATAGATCTCTGGTCTTACAACGCGGAGCTTCTTGTTGCCCTGGAGAACCAACATACAATTGATTTAACXGACTCAGAXATGAXCAAACTGTTCGAAAGAACAAGAAGGCAAC
1290 1300 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
HA Sequence 1
HA Sequence 2
HA Sequence 3
HA Sequence 4
HA Sequence 5
HA Sequence 6
HA Sequence 7
HA Sequence 8
Majority TGCGGGAAAATGCTGAGGACATGGGCAATGGTTGCTTCAAAATATACCACAAATGTGACAATGCCTGCATAGGATCAATCAGAAATGGAACTTATGACCATGATATATACAGAAACGAGGCATTAAACAATCGGTTCCAGATCAAAGGTGTTCAGCTAAA
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
HA Sequence 1
HA Sequence 2
HA Sequence 3
HA Sequence 4
HA Sequence 5
HA Sequence 6
HA Sequence 7
HA Sequence 8
Majority GTCAGGATACAAAGATTGGATCCTATGGATTTCCTTTGCCATATCATGCTTTTTGCTTTGTGTTGTTCTGCTGGGGTTCATTATGTGGGCCTGCCAAAAAGGCAACATTAGGTGCAACATTTGCATTTGAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700 1710 1720 1730 1740 1750 1760
HA Sequence 1
HA Sequence 2
HA Sequence 3 GTGCATTAATTAAAAACACCCTTGTT
HA Sequence 4 GTACATTAATTAAAAACACC
HA Sequence 5 GTGCATTAATTAAAAACACCCTTGTTTTCT
HA Sequence 6
HA Sequence 7
HA Sequence 8


Majority ------------------ATGAATCCAAATCAAAAGATAATAACAATTGGCTCTGTTTCTCTCATTATTGCCACAATATGCTTCCTTATGCAAATTGCCATCCTGGTAACTACTGTAACATTGCATTTCAAGCAGCATGACTGCAACTCCCCCCCAAAC
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
NA Sequence 1 ------------------.....C.....G.....A.....A.....
NA Sequence 2 ------------------.....C.....C.....
NA Sequence 3 ------------------.....C.....C.....T.....
NA Sequence 4 ------------------.....C.....T.....T....A.....A.....A.....
NA Sequence 5 ------------------.....C.....C.....
NA Sequence 6 NNNNNNNNNNNNNNNNNNN.....C.....T.....G.....T....T..T.....
NA Sequence 7 ------------------.....A.....A.....
NA Sequence 8 ------------------.....G.....C.....G.....AT....A.....
Majority AACCAAGTAATGCTGTGTGAACCAACAATAATAGAAAGAAACACTACAGAXATTGTGTATXTGACCAACACCACCATAGAAAAGGAAATATGCCCCAAACTAGCAGAATACAGAAATTGGTCAAAGCCGCAATGTAACATXACAGGATTTGCACCTTTTT
170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320
NA Sequence 1 .....A.....G.....C.....T.....G.....G.....T.....
NA Sequence 2 .....A.....T.....C.....
NA Sequence 3 .....A.....T.....C.....
NA Sequence 4 .....A.....G.....T.....G..A.....T.....C.....T.....
NA Sequence 5 .....A.....T.....C.....
NA Sequence 6 .....TG.....G.....C.....T.....T.....C...T.....T.....T.....
NA Sequence 7 .....A.....C.....G.....A.....C.....
NA Sequence 8 .....TA.....G..A.....C.....G.....A.....A..T.....G....
Majority CTAAGGACAATTCGATTCGGCTTTCCGCTGGTGGGGACATCTGGGTGACAAGAGAACCTTATGTGTCATGCGATCCTGACAAGTGTTATCAATTTGCCCTTGGGCAGGGAACAACACTAAACAACAGGCATTCAAATGACACTGTACATGATAGGACCCC
330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480
NA Sequence 1 .....T..C.....T.....A.....T.G.....A.....T....
NA Sequence 2 .....T.....C.....
NA Sequence 3 .....T.....C.....
NA Sequence 4 .....A.....T.....T.....G.A.....A.....
NA Sequence 5 .....T.....C.....
NA Sequence 6 .....A.....G.....A.....
NA Sequence 7 .....T.....G.....G.....G.....
NA Sequence 8 .C.....A.....T.....A.....A.....
Majority TTACCGAACCCTATTGATGAATGAATTGGGTGTTCCATTXCATTTGGGAACCAGGCAAGTGTGCATXGCATGGTCCAGCTCAAGTTGTCACGATGGAAAAGCATGGCTGCATGTTTGTATAACTGGGGATGATAAAAATGCAACTGCTAGCTTCATTTAC
490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640
NA Sequence 1 .....T.....A.....C.....G.....C..C.....C..C.....T.....
NA Sequence 2 .....C.....G.....G.....
NA Sequence 3 .....C.....G.....G.....
NA Sequence 4 ...T.....T.....A.....G.....A.....C.....A.....
NA Sequence 5 .....C.....G.....G.....
NA Sequence 6 .....C.....T.....A.....T.....A.....
NA Sequence 7 .....C..C.....A.....T.....
NA Sequence 8 ...T.....G.....T.....A.....A.....G.....C.....G.....
Majority AATGGGAGGCTTGTAGATAGTATTGGTTCATGGTCCAAAAATATACTCAGAACCCAGGAGTCGGAATGCGTTTGTATCAATGGAACTTGTACAGTAGTAATGACTGATGGGAGCGCTTCAGGAAAAGCTGATACTAAAATACTATTCATTGAGGAGGGGA
650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800
NA Sequence 1 .....A.....G.....T.....G.....
NA Sequence 2 .....
NA Sequence 3 .....
NA Sequence 4 .....C.....C.....G.....
NA Sequence 5 .....
NA Sequence 6 .....A.....A..A.....C.....A.....
NA Sequence 7 .....
NA Sequence 8 G.....A..C.....G.....T.....G.....T.....
Majority AAATCGTTCATATTAGCACATTGTCAGGAAGTGCTCAGCATGTCGAGGAGTGCTCCTGTTATCCTCGATATCCTGGTGTCAGATGTGTCTGCAGAGACAACTGGAAAGGCTCCAAXAGGCCCATCGTAGATATAAATGTAAAGGATTATAGCATTGTTTC
810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960
NA Sequence 1 .G.....T.....A.G.....C...
NA Sequence 2 .....A.....C.....A.....
NA Sequence 3 .....A.....C.....A.....
NA Sequence 4 .G.....T.....T.....T.....A.....
NA Sequence 5 .....A.....C.....
NA Sequence 6 .....C.....T.....C.....T.....
NA Sequence 7 ....A.....T.....T.....
NA Sequence 8 .....C.....T.....T.....
Majority CAGTTATGTATGCTCXGGACTTGTTGGAGACACACCCAGAAAAAACGACAGCTTCAGCAGTAGCCATTGCCTAGATCCTAACAATGAGGAAGGTGGTCATGGAGTGAAAGGCTGGGCCTTTGATGATGGAAATGACGTGTGGATGGGAAGAACGATCAGC
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100 1110 1120
NA Sequence 1 .....A.....T.....A.....G.....G.....
NA Sequence 2 ..A.....G.....G.....
NA Sequence 3 ..A.....G.....G.....
NA Sequence 4 .....A.....G.....G.....TT.....A.....G.....G..A....A.
NA Sequence 5 .....G.....G.....
NA Sequence 6 A.....A.....T.....A.....G.....
NA Sequence 7 .....G.....C.....A.....
NA Sequence 8 ...C.....G.....A.....A.C.....A.....GA.....G.....


Majority GAGAAGTTACGCTCAGGTTATGAAACCTTCAAAGTCATCGAAGGCTGGTCCAAACCTAATTCCAAATTACAGACAAATAGGCAAGTCATAGTTGAAAGAGGTAAAAGGTCCGGTTATTCTGGTATTTTCTCCGTTGAAGGCAAAAGCTGCATCAATCGGT
1130 1140 1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280
NA Sequence 1 ...G....T....T.................G......A.............C.....................................A......C...........C......G........A......A.....
NA Sequence 2 ....C.....
NA Sequence 3 ....C.....
NA Sequence 4 ....C........T.......................T..............G....C.....................................A...G.C.....................................T.....
NA Sequence 5 ....C.....
NA Sequence 6 ...G...C.....................................C.....
NA Sequence 7 ..T.....................................T.....
NA Sequence 8 .....A.............................T.....................C........G....T..................C..C......G.T..................C........T.....
Majority GCTTTTATGTXGAGTTGATAAGGGGAAGGAAAGAGGAAACTAAAGTCTGGTGGACCTCAAACAGTATTGTTGTGTTTTGTGGCACCTCAGGTACATATGGAACAGGCTCATGGCCTGATGGGGCGGAXXXXXXXXXXXXXXXXXXXXXX
1290 1300 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420
NA Sequence 1 ..........G.................A...........C.....................................T.....................................CATCAA
NA Sequence 2 ....A.....................................CATCAA
NA Sequence 3 ....A.....
NA Sequence 4 ..........G.....A.....CATC
NA Sequence 5 ....A.....
NA Sequence 6 ..........G.....T.....
NA Sequence 7 ..........A.....C....
NA Sequence 8 ..........G.....................C........G.....CATCAATCTCATGCCTATATAA